Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene

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Secreted parasitism proteins encoded by parasitism genes expressed in esophageal gland cells mediate infection and parasitism of plants by root-knot nematodes (RKN). Parasitism gene *16D10* encodes a conserved RKN secretory peptide that stimulates root growth and functions as a ligand for a putative plant transcription factor. We used *in vitro* and *in vivo* RNA interference approaches to silence this parasitism gene in RKN and validate that the parasitism gene has an essential function in RKN parasitism of plants. Ingestion of *16D10* dsRNA *in vitro* silenced the target parasitism gene in RKN and resulted in reduced nematode infectivity. *In vivo* expression of 16D10 dsRNA in *Arabidopsis* resulted in resistance effective against the four major RKN species. Because no known natural resistance gene has this wide effective range of RKN resistance, bioengineering crops expressing dsRNA that silence target RKN parasitism genes to disrupt the parasitic process represents a viable and flexible means of developing novel durable RKN-resistant crops and could provide crops with unprecedented broad resistance to RKN.

double-stranded RNA | RNA interference | broad resistance | plant-parasitic nematode

Root-knot nematodes (RKN, *Meloidogyne* species) are the most economically important group of plant-parasitic nematodes worldwide, attacking nearly every food and fiber crop grown (1). Four common RKN species (*M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*) account for 95% of all RKN infestations in agricultural land, with *M. incognita* being the most important species (2). These highly successful pathogens infect >1,700 host plant species and are devastating global agricultural pests (1). The most cost-effective and sustainable method for reducing RKN damage to food and fiber crops is to develop resistant plants that suppress nematode development and reproduction (3, 4). However, only a limited number of plant species are resistant to RKN, and there are many crops for which appropriate resistance loci have not been identified (4, 5). As with other plant resistance genes, the function of available RKN resistance genes involves recognition of specific RKN biotypes, rendering crops vulnerable to selection for virulent field populations (6, 7).

Secreted proteins encoded by parasitism genes expressed in nematode esophageal gland cells are critical for the invading RKN to transform selected root vascular cells into elaborate feeding cells, called giant-cells (8–10). We recently reported that a peptide (16D10) secreted from the subventral esophageal gland cells of parasitic second-stage juveniles (J2) of RKN affects root growth by directly interacting with a specific domain of a putative plant SCARECROW-like transcription factor (11). The secreted 16D10 parasitism peptide is conserved across RKN species and appears to mediate an early signaling event in RKN–host interactions.

RNAi, first characterized in *Caenorhabditis elegans* (12), has evolved into a powerful gene silencing tool for analysis of gene function in a wide variety of organisms (13). In plants and nematodes, introducing or expressing dsRNA triggers the target gene-specific RNAi pathway (14), including RNAi of target genes at sites distal to the location of dsRNA that is ingested by nematodes (15). RNAi effects on preparasitic J2 of plant-parasitic nematodes have been achieved *in vitro* by incubating J2 in solutions to stimulate dsRNA ingestion through the nematode's mouth spear (stylet) outside of the host plant (16, 17). Inoculation of plants with cyst nematode juveniles that have ingested dsRNA *in vitro* resulted in partial silencing of the target genes and fewer nematodes being recovered from infected plants compared with plants inoculated with control-treated nematodes (16, 18, 19).

Here, we describe utilization of *in vitro* and *in vivo* RNAi approaches to silence the parasitism gene *16D10* in RKN and validate that the parasitism gene has an essential role in RKN parasitism of plants. Ingestion of *16D10* dsRNA *in vitro* silenced *16D10* in RKN and resulted in reduced nematode infectivity. *In vivo* expression of *16D10* dsRNA in *Arabidopsis* resulted in resistance effective against the four major RKN species. Significantly, no natural root-knot resistance gene has this effective range of RKN resistance. Therefore, our results of *in planta* RNAi silencing of parasitism gene *16D10* in RKN could lead to the development of crops with broad resistance to this destructive pathogen.

Results and Discussion

***In Vitro* RNAi of *16D10*.** Almost 100% of RKN J2 stimulated *in vitro* to ingest solutions containing truncated or full-length dsRNA of parasitism gene *16D10* displayed an ingestible fluorescent marker in the lumen of the alimentary canal (Fig. 1*A*). Real-time quantitative RT-PCR and ELISA analyses revealed that the ingestion of truncated or full-length *16D10* dsRNA by *M. incognita* J2 led to a 93–97% reduction of *16D10* transcripts (Fig. 1*B*) and a 65–69% reduction of the 16D10 peptide (Fig. 1*C*) in the treated nematodes compared with control-treated nematodes. Inoculation of *Arabidopsis* roots with *M. incognita* J2 that had ingested *16D10* dsRNA *in vitro* resulted in suppression of nematode development (reproduction) by 74–81% and gall formation lower in number and smaller in size when compared with inoculations with control-treated nematodes (Fig. 1 *D* and

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Abbreviations: J2, second-stage juveniles; J3, third-stage juveniles; Res, resorcinol; RKN, root-knot nematodes.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ841121–DQ841123).

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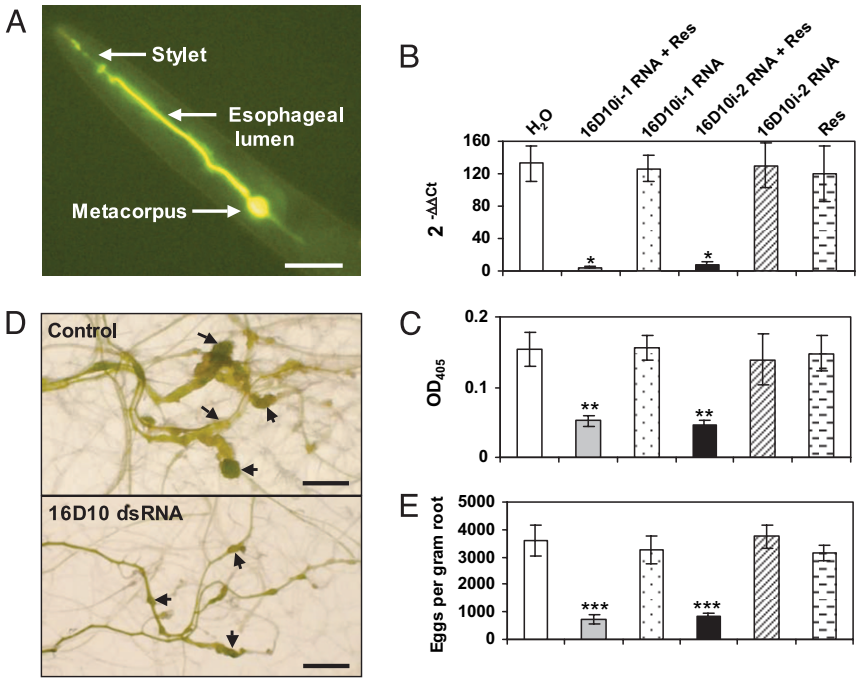

Fig. 1. RNAi silencing of *16D10* in preparasitic *M. incognita* J2. (*A*) Fluorescence microscopy showing ingestion of FITC in the treated J2. (Scale bar, 10 μm.) (*B*) Real-time RT-PCR analysis of *16D10* transcript abundance in the FITC-labeled, transgenic J2 after soaking with short or full-length dsRNA molecules (16D10i-1 RNA or 16D10i-2 RNA) of *16D10* and Res. Controls are J2 soaked in H_2O only, dsRNA without Res (bars labeled 16D10i-1 RNA and 16D10i-2 RNA), and Res without dsRNA (bar labeled Res), respectively. $2^{-\Delta\Delta Ct}$ represents the amount of *16D10* that is normalized to an endogenous reference (actin) and relative to a calibrator (*16D10*) from the adult female stage, which has the lowest expression level of *16D10*. $\Delta\Delta C_t = (\Delta C_{t\text{-}16D10} - \Delta C_{t\text{-}16D10adult})$; $\Delta C_{t\text{-}16D10} = (C_{t\text{-}16D10} - C_{t\text{-}actin})$; $\Delta C_{t\text{-}16D10adult} = (C_{t\text{-}16D10adult} - C_{t\text{-}actin})$. Each bar value represents the mean ± SD of triplicate experiments (Student's *t* test; *, $P < 0.001$ versus controls). (*C*) ELISA analysis of 16D10 protein in the treated J2 using the purified 16D10 peptide antiserum (11). Ten micrograms of total extracts of the treated J2 is used in each bar. Each bar value represents the mean ± SD of triplicate experiments (Student's *t* test; **, $P < 0.01$ versus controls). (*D*) Wild-type *Arabidopsis* roots inoculated with control J2 (*Upper*) or full-length *16D10* dsRNA treated J2 (*Lower*) showing numerous larger galls (*Upper*) or fewer small galls (*Lower*) 7 weeks after inoculation, respectively. RKN infection sites are indicated by arrows. (Scale bars, 10 mm.) (*E*) Reproduction (eggs per gram root) of each of treated *M. incognita* on wild-type *Arabidopsis* roots. Each bar value represents the mean ± SD of $n = 36$ (Student's *t* test; ***, $P < 0.01$ versus controls).

E). This significant reduction in the infectivity of the *16D10* dsRNA-treated J2 indicated that *16D10* is an essential parasitism gene for *M. incognita* infection of plants. The combined data provided empirical support for the feasibility of *in vivo* targeting of *16D10* in *M. incognita* by RNAi.

***In Vivo* RNAi of *16D10*.** Vectors designed to direct the expression of hairpin dsRNA within host plants (20) may be used to promote *in vivo* dsRNA ingestion and silencing of target nematode genes as a functional analysis of nematode parasitism genes and to potentially create novel transgenic crops that are resistant to nematodes (10). Plants display posttranscriptional gene silencing that operates in the same manner as RNAi in nematodes, by dicer-mediated digestion of dsRNA molecules into siRNAs of ≈21 nucleotides (21). Available siRNAs in plant cells would be of a size to navigate the size exclusion limit of ≈40 kDa (≈62-bp dsRNA) required for ingestion through the RKN stylet *in planta* (10). To test the potential of *in vivo* RNAi, we generated two transgenic *Arabidopsis* homozygous T_2 lines to produce truncated or full-length *16D10* dsRNA molecules driven by the cauliflower mosaic virus 35S promoter using the pHANNIBAL vector (20). Also, a transgenic line originating from the blank transformation vector was generated as a control. No significant morphological differences were found in these transgenic lines when compared with wild-type *Arabidopsis*. RNA blot analysis showed that the *16D10* dsRNAs were transcribed in the transgenic lines and processed by *Arabidopsis* cells to ≈21-bp siRNA (Fig. 2*A*). The processing of constitutively expressed *16D10* dsRNA in transgenic *Arabidopsis* provides *16D10* siRNA molecules for ingestion by parasitic stages of RKN and subsequent RNAi of parasitism gene *16D10* in the subventral esophageal glands of the nematode. The subventral esophageal glands are very active in the infective and parasitic J2 of the RKN until the J2 molts to the third-stage juvenile (J3) life stage at ≈11–13 days after root penetration. At this time, the J2 has induced differentiation of the giant-cells, commenced feeding, and grown to become sedentary, i.e., the initial and critical stages of giant-cell formation have been completed. The subventral esophageal glands subsequently become less functional, which ties their roles firmly to the critical early stages of parasitism.

Potential effects of the host-generated *16D10* dsRNA (siRNA) molecules on plant infection by RKN were assessed in agar plate assays using the transgenic *Arabidopsis* lines with each of the four major RKN species: *M. incognita*, *M. javanica*, *M. arenaria*, or *M. hapla*. Four weeks after inoculation with RKN, control transgenic lines from the transformation with the empty vector had numerous large galls, whereas *16D10* dsRNA transgenic lines showed a 63–90% reduction in the number of galls as well as an overall decrease in gall size (Fig. 2*B*) compared with the vector-transformed line. Reproduction assays revealed a 69–93% reduction in the number of RKN eggs per gram root in the *16D10* dsRNA transgenic lines when compared with the infected control plants (Fig. 2*C*). These RNAi results convincingly demonstrate that (*i*) parasitism gene *16D10* has an essential function in RKN parasitism of *Arabidopsis*, and that (*ii*) *in planta* delivery of RNAi of *16D10* in RKN results in broad RKN resistance.

DNA blots showed that parasitism gene *16D10* is highly conserved in RKN species (11). Homologues to the *M. incognita* *16D10* (GenBank accession no. DQ087264), amplified by using

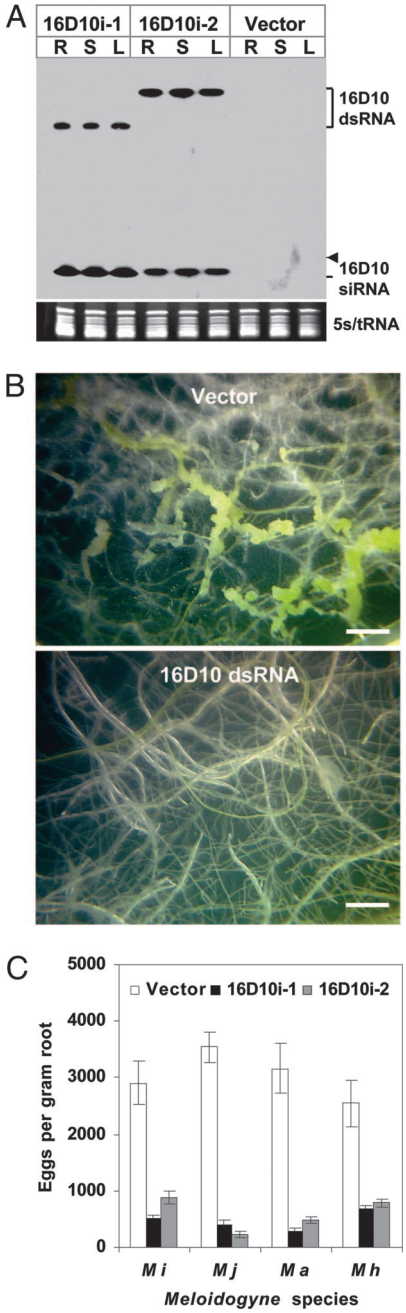

Fig. 2. Overexpression of *16D10* dsRNA in *Arabidopsis*. (*A*) RNA blots for the expression of *16D10* dsRNA in two *16D10* dsRNA transgenic homozygous T_2 lines (16D10i-1 and 16D10i-2) and the absence of *16D10* dsRNA expression in one vector-transformed homozygous T_2 line (Vector). R, root; S, stem; L, leaf. An ≈21-nt *16D10* siRNA was detected in the two *16D10* dsRNA transgenic lines. Arrowhead indicates the position of a 25-base DNA oligonucleotide. Ethidium bromide-stained gel (before transfer) in the zone corresponding to 5S RNA and tRNA is shown at the bottom. (*B*) RNAi inhibition of *M. incognita* infection of *A. thaliana*. Control vector-transformed line with numerous galls (*Upper*) and *16D10* dsRNA transgenic line (16D10i-1) showing no galls (*Lower*) 8 weeks after inoculation. (Scale bars, 10 mm.) (*C*) Reproduction (eggs per gram root) of four *Meloidogyne* species (*Mi*, *M. incognita*; *Mj*, *M. javanica*; *Ma*, *M. arenaria*; *Mh*, *M. hapla*) on transgenic *A. thaliana* expressing *16D10* dsRNA is significantly decreased compared with control plants. Each bar value represents the mean ± SD of $n = 24–48$ (Student's *t* test, $P < 0.001$ versus control).

the primers 16D10GF and 16D10GR (Table 1 and Fig. 4, which are published as supporting information on the PNAS web site), from *M. javanica*, *M. arenaria*, and *M. hapla* (accession nos. DQ841121-DQ841123) showed a 95–98% nucleotide identity, whereas the predicted peptides were identical in all four species. Because the transgenic *Arabidopsis* lines expressing *16D10* dsRNA molecules were resistant to these four RKN species, we infer that secreted 16D10 peptide is a fundamental signaling molecule for regulating RKN–host interactions. This potential vital role as a signaling molecule for the 16D10 peptide is further supported by parasitism gene *16D10* being strongly expressed in the subventral esophageal gland cells of parasitic J2 during the time when the parasitized root cells are differentiating into giant-cells and the binding of the secreted peptide to a putative plant transcription factor domain (11).

RNAi Targeting of *16D10* Overexpessed in Plants. For additional evidence supporting the RNAi silencing of parasitism gene *16D10*, we conducted RNAi silencing of *16D10* in *Arabidopsis* by crossing the transgenic *Arabidopsis* line overexpressing *16D10* with the *16D10* dsRNA transgenic line to generate F_1 hybrid lines. Overexpression of *16D10* in *Arabidopsis* stimulates root growth by 85% (11), whereas root growth on RKN-resistant *16D10* dsRNA transgenic *Arabidopsis* line (16D10i-1) was comparable to root growth on wild-type plants. In RNA blot analysis, the *16D10* mRNA present in the maternal *16D10*-overexpressing transgenic line was not detected in the F_1 hybrid line, but a higher level of *16D10* siRNA was detected in the hybrid line when compared with the paternal *16D10* dsRNA transgenic line (Fig. 3*A*). Complete RNAi silencing of *16D10* expression in the F_1 hybrid line restored the *16D10*-stimulated root growth phenotype of the maternal *16D10*-overexpressing transgenic line to wild-type normal root growth phenotype [mean root lengths were 61 ± 14 mm in the *16D10* maternal transgenic line, 35 ± 6 mm in the *16D10* dsRNA paternal transgenic line, and 33 ± 10 mm in the hybrid line, $n = 30$ per line (Student's *t* test, $P < 0.01$)] (Fig. 3 *B* and *C*). The effects of this *in planta* silencing of overexpressed *16D10* confirm that the host-generated *16D10* dsRNA (siRNA) can trigger RNAi of *16D10* to subsequently interfere with the function of the RKN 16D10 parasitism peptide in plants.

Although RNAi is considered to be highly gene-specific, siRNAs can induce "off-target" gene silencing effects (22). Even though *16D10* dsRNAs expressing in the transgenic *Arabidopsis* lines were processed to siRNA, no off-target effects (change in phenotype) were observed with the transgenic *Arabidopsis* lines (Fig. 3*B*). *16D10* has a novel nucleotide sequence without homologues (>19-nt identity) in the *Arabidopsis* genome/EST databases (11), suggesting the absence of a potential gene target in *Arabidopsis* for the *16D10* siRNA. In addition, no significant differences were found in the number of cysts or eggs per gram of root between the *16D10* dsRNA transgenic lines and the wild-type control *Arabidopsis* when inoculated with the beet cyst nematode *Heterodera schachtii* (data not shown). These results show that the RNAi effect is specific for the *16D10* parasitism gene in RKN and does not target other nematode genes.

A report that describes the effects of host-derived RNAi on plant infection by RKN takes the approach of targeting splicing factor and integrase genes essential to nematode cellular and developmental processes, although no siRNA data were presented (23). Our approach of targeting parasitism genes unique to RKN–host interactions may prove to be a more desirable strategy because it targets parasitism directly and appears to minimize the threat of off-target effects. However, only extensive study of different approaches in the future will be able to identify the most effective and practical means of controlling plant-parasitic nematodes using this technology.

In summary, we report that targeting the RKN parasitism gene *16D10* for silencing by expressing dsRNA in transgenic *Arabidopsis* resulted in transgenic plants that were resistant to multiple RKN species. These results validate that this parasitism gene is

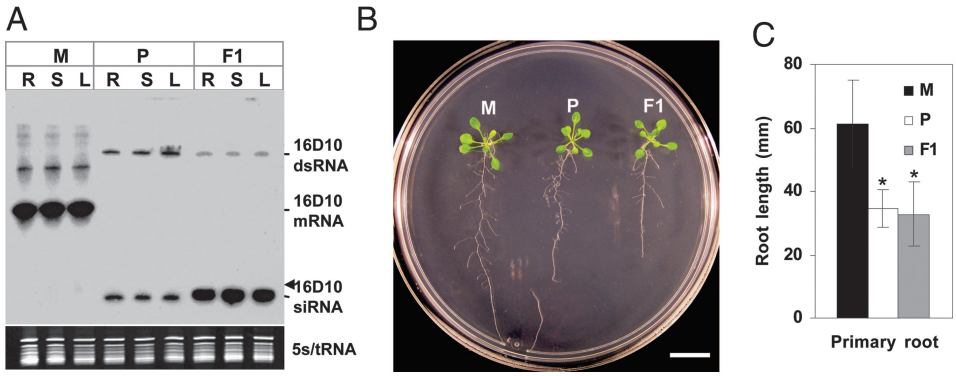

Fig. 3. RNAi silencing of *16D10* in *Arabidopsis*. (*A*) RNA-blot analysis of silencing of *16D10* mRNA in *A. thaliana* hybrid line. The maternal plant (M, the *16D10* transgenic homozygous T_2 line L17) expressing *16D10* mRNA and the paternal plant (P, the *16D10* dsRNA transgenic homozygous T_2 line 16D10i-1) expressing *16D10* dsRNA (siRNA) are shown, but the expression of *16D10* mRNA in the hybrid line (F_1) is silenced. R, root; S, stem; L, leaf. Arrowhead indicates the position of a 25-nt DNA oligonucleotide. Ethidium bromide staining in the zone corresponding to 5S RNA and tRNA is shown. (*B*) Seedlings of the *16D10* hybrid line (*Right*), the *16D10* transgenic homozygous T_2 line L17 (*Left*), and the *16D10* dsRNA transgenic homozygous T_2 line 16D10i-1 (*Center*) 12 days after germination. (Scale bar, 10 mm.) (*C*) Primary root length 12 days after germination. Each bar value represents the mean ± SD of $n = 30$ (Student's *t* test, $P < 0.01$ versus the maternal plant; *, $P > 0.1$).

essential for RKN parasitism of plants, and more significantly, they led to the availability of a resistance gene effective against the world's most damaging plant-parasitic nematodes with an effective range of resistance not conditioned by any natural RKN resistance gene. Therefore, our results of *in planta* RNAi silencing of parasitism gene *16D10* in RKN could lead to the development of transgenic crops with effective broad host resistance to this agriculturally important pathogen. Equally important, our approach provides a strategy for developing RKN-resistant crops for which natural resistance genes do not exist.

Materials and Methods

Nematodes. RKN were cultured on susceptible tomato plants. Eggs and preparasitic J2 were collected as described in refs. 3 and 24. Adult females of *M. incognita* were hand-picked from infected tomato roots.

***In Vitro* RNAi of *16D10*.** Forty-two base pair (the peptide-coding region, 16D10i-1) and 271-bp sequences (the full-length sequence excluding AT-rich regions at the 5′ and 3′ ends, 16D10i-2) of parasitism gene *16D10* were amplified from the full-length cDNA clone by using the primers 16D10T7F1 and 16D10T7R1 and 16D10T7F2 and 16D10T7R2 (Table 1 and Fig. 4), respectively, each of which incorporates the RNA primer site T7. The gel-purified PCR products were used as templates for synthesis of sense and antisense *16D10* RNAs in a single reaction *in vitro* by using a MEGAscript RNAi kit (Ambion, Austin, TX) according to the manufacturer's instructions. Freshly hatched J2s (≈10,000) of *M. incognita* were soaked in 0.25× M9 buffer (25) containing 1 mg/ml dsRNA, 1% resorcinol (Res), 0.1 mg/ml FITC isomer I, 0.05% gelatin, and 3 mM spermidine and incubated for 4 h in the dark at room temperature on a rotator. Res was used to stimulate uptake of the dsRNA. Control samples were incubated in the same solution but without Res or dsRNA or both (with H_2O only). After soaking, nematodes were thoroughly washed five times with nuclease-free water by centrifugation and treated nematodes were observed with an fluorescence microscope (Olympus, Melville, NY) to monitor uptake of FITC. The FITC-labeled transgenic J2 were incubated in nuclease-free water for 24 h at room temperature to allow for turnover of 16D10 protein and then assayed by using real-time quantitative RT-PCR (500 J2), ELISA, and infection of host roots (50 J2 per plant) to determine RNAi silencing of the nematode endogenous *16D10*.

mRNA was extracted from homogenized females (300 nematodes) or treated J2 of *M. incognita* and converted into first-strand cDNA according to Huang *et al.* (9, 11). Real-time quantitative RT-PCR was performed in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Core Reagents (Applied Biosystems). The amplifications were conducted as described in ref. 26 using the 1:50 diluted first-strand cDNA with the primer pairs 16D10RTF and 16D10RTR or MIAF and MIAR (Table 1 and Fig. 4) to amplify a *16D10* region and a control region of the *M. incognita* actin gene (GenBank accession no. BE225475). Expression of *16D10* relative to the endogenous actin control was determined using the $\Delta\Delta C_t$ method described in the ABI PRISM 7700 Sequence Detection System Bulletin 2 (Applied Biosystems).

Nematode proteins were extracted from homogenized *M. incognita* J2 as described in ref. 24. ELISA analysis of 16D10 peptide in the treated J2 was performed as described in ref. 11 using BSA as a negative control.

dsRNA-treated *M. incognita* J2 and control nematodes were suspended in 0.001% chlorhexidine diacetate for 30 min and then sterilized with 0.01% $HgCl_2$ for 7 min followed by three 2-min washes with sterile H_2O. Twelve *Arabidopsis thaliana* wild-type Col-0 plants in each of three repeats were *in vitro* cultured in Gamborg's B-5 medium (Sigma, St. Louis, MO) for 3 weeks and inoculated with 50 sterilized J2 in each plant at the root tips (10–15 J2 per root tip). The number and size of galls on the infected roots were analyzed 3 weeks after inoculation. The infected roots were stained with acid fuchsin, and RKN reproduction was assayed by determining the number of eggs per gram of roots 7 weeks after inoculation as described in ref. 3.

***In Vivo* RNAi of *16D10*.** The sense and anti-sense cDNA sequences (42-bp and 271-bp) of parasitism gene *16D10* used in the *in vitro* RNAi experiments were amplified from the full-length cDNA clone with the gene-specific primers (Table 1 and Fig. 4) that introduced XhoI, KpnI, ClaI, or XbaI restriction sites, cloned into the XhoI-KpnI sites and the ClaI-XbaI sites of pHANNIBAL (20) to generate pHANNIBAL(16D10i-1) and pHANNIBAL(16D10i-2), respectively, and confirmed by sequencing. The constructs made in pHANNIBAL were subcloned as NotI fragments into the binary vector pART27 to produce highly effective intron-containing "hairpin" RNA silencing constructs [pART27(16D10i-1) and pART27(16D10i-2)]. The pART27-derived constructs including the empty vector pART27 as a control were introduced into

Agrobacterium tumefaciens C58C1 by electroporation and transformed into *A. thaliana* wild-type Col-0 plants by the floral dip method (27). Segregation analysis of kanamycin resistance identified transgenic homozygous T_2 lines (16D10i-1 and 16D10i-2), and PCR analysis was used to confirm the presence of the transgene. Sixteen plants from each transgenic line in each of the three repeats were *in vitro* cultured on Gamborg's B-5 plates for 3 weeks and then inoculated with ≈500 surface-sterilized eggs of *M. incognita*, *M. javanica*, *M. arenaria*, or *M. hapla* placed near the roots for each plant. The RKN galling and reproduction assays were performed as described above.

RNA blots were conducted as described in ref. 21 with some modifications. Total RNA was extracted from equivalent amounts of plant tissues (0.1 g) of each transgenic line, and small RNAs were enriched by using the *mir*Vana miRNA isolation kit (Ambion) according to the manufacturer's instructions and then treated with DNA-*free* (Ambion) to remove any DNA contaminants. RNA samples (15 μl) were separated on 0.5× Tris-borate EDTA/7 M urea/15% PAGE, stained with ethidium bromide, and electro-transferred onto Hybond-XL nylon membranes (Amersham Biosciences, Piscataway, NJ). Parasitism gene *16D10* probe was labeled from the 271-bp gel-purified PCR product of *16D10* with [α-^{32}P]dCTP by using the RadPrime DNA labeling system (Invitrogen, Carlsbad, CA). Hybridizations were performed in 50% formamide, 5× SSC, 5× Denhardt's solution (0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% BSA), 0.5% SDS, and 20 ng/ml sheared, denatured salmon sperm DNA at 30°C for 16 h, followed by two 10-min washes in 2× SSC/0.1% SDS solution at room temperature. The membranes were washed twice at 50°C with 0.5× SSC/0.1% SDS solution for 40 min and exposed to x-ray films overnight at −80°C.

RNAi Targeting of *16D10* Overexpressed in Plants. The *16D10* transgenic *Arabidopsis* homozygous T_2 line L17 (11) and the *16D10* dsRNA transgenic homozygous T_2 line 16D10i-1 were used as maternal and paternal plants, respectively, to generate hybrid lines. The F_1 hybrid lines containing both *16D10* and *16D10* dsRNA expression cassettes were verified by PCR analysis. Root-growth assay was performed as described in ref. 11. RNA blot analysis for expression of *16D10* in the hybrid lines and the parental lines was carried out as described above.

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